NK1-receptor expressing paraventricular nucleus neurones modulate daily variation in heart rate and stress induced changes in heart rate variability.

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Subject area: Cardiovascular control

New findings

- **What is the central question of this study?**

  There is a substance P dependent pathway projecting from the PVN to the spinal cord; associated with cardiovascular control. Do these NK1-receptor expressing neurones influence the cardiovascular system and are they involved in the cardiovascular response to stress?

- **What is the main finding and its importance?**

  HRV analysis showed increases in LF/HF ratio in response to psychological stress, consistent with an increase in sympathetic activity. Lesioning NK1-receptor expressing neurones in the PVN abolished this response and resulted in a 3 hour shift in the daily variation of heart rate. This shows for the first time the importance of NK1-receptor expressing neurones in the PVN in cardiovascular control.

Abstract

The paraventricular nucleus of the hypothalamus (PVN) is an established centre of cardiovascular control, receiving projections from other nuclei of the hypothalamus such as the dorsomedial hypothalamus and the suprachiasmatic nucleus. The PVN contains a population of "pre-autonomic neurones" which project to the intermediolateralis of the spinal cord and increase sympathetic activity, blood pressure and heart rate. These spinally projecting neurones express a number of membrane receptors including GABA and substance P NK1 receptors. Activation of NK1 expressing neurones increases heart rate, blood pressure and sympathetic activity. However, their role in the pattern of overall of cardiovascular control remains unknown. In this work we use specific saporin lesion of NK1 expressing PVN rat neurones with SSP-SAP and telemetrically measure resting heart rate and heart rate variability (HRV) parameters in response to mild psychological stress. The HRV parameter "low frequency/high frequency ratio" is often used as an indicator of sympathetic activity

and is significantly increased with psychological stress in control rats (0.84 ± 0.14 to 2.02 ± 0.15; $p<0.001$; n=3). We find the stress induced increase in this parameter to be blunted in the SSP-SAP lesioned rats (0.83 ± 0.09 to 0.93 ± 0.21; $p>0.05$; n=3). We also find a shift in daily variation of heart rate rhythm and conclude that NK1 expressing PVN neurones are involved with coupling of the cardiovascular system to daily heart rate variation and the sympathetic response to psychological stress.

Introduction

A population of paraventricular nucleus (PVN) hypothalamic parvocellular neurones projects directly to sympathetic control "centres" of the medulla and spinal cord (Pyner & Coote, 2000) and modulates heart rate (HR) and blood pressure (BP) (Coote, 2007). The activity of these neurones becomes elevated in heart failure as their tonic inhibitory GABA-ergic input becomes reduced (Pyner, 2014). Although this pathway is therefore of huge importance to cardiovascular medicine, there is no consensus as to its specific role in cardiovascular control. Theories to date include mediation of the cardiovascular response to stress, control of blood volume and circadian changes in HR. In our previous work we have shown that these neurones can be controlled by tachykinin neuropeptides (Womack *et al.*, 2007). In this work we report the effect of selective lesion of PVN neurokinin 1 (NK1) receptor expressing neurones on heart rate and heart rate response to psychological stress in rats.

A number of neurotransmitters and modulators are known to act on spinally projecting neurones, including GABA, glutamate, nitric oxide and adenosine (Pyner, 2009; Nunn *et al.*, 2011; Affleck *et al.*, 2012). However, recent focus has been on the tachykinin family of neuropeptides (including substance P, SP), since evidence suggests that the tachykinins (including SP), especially NK1 receptor activating ligands (Culman & Unger, 1995; Culman *et al.*, 2010; Tauer *et al.*, 2012), are important for the central control of mean arterial blood pressure (Culman & Unger, 1995; Culman *et al.*, 2010). In our own recent work we characterised, *in vitro*, an SP dependent pathway linking the PVN to another important cardiovascular control centre in the hypothalamus; the dorsomedial hypothalamus (DMH) (Womack & Barrett-Jolley, 2007), and an associated SP activated (sympathostimulatory) pathway projecting from the PVN to the intermediolateral spinal cord (Womack *et al.*, 2007).

68 The PVN has been known to be a site for integration of the hormonal response to stress (Herman &
69 Cullinan, 1997) for some time, and it was recently confirmed that a proportion of the noxious stress
70 response (subcutaneous formalin) was sensitive to intracerebroventricular (ICV) application of a
71 selective NK1 and NK2 antagonist (Culman *et al.*, 2010). Furthermore, psychological stress (using
72 elevated plus maze test) is markedly reduced in rats given ICV injection of a selective NK1 receptor
73 antagonist. In the same study stress-induced c-Fos expression within the PVN is lower after
74 pharmacological blockade of the NK1 receptor (Ebner *et al.*, 2008). Reduced c-Fos expression in the
75 PVN is also seen in NK1R-/- mice subjected to same stressor (Santarelli *et al.*, 2002). Levels of the
76 stress hormone cortisol are decrease compared to their wild-type counterparts as a result of this
77 stress test (Santarelli *et al.*, 2001). However, the theory that the PVN is generally important for the
78 cardiovascular response to stress (Dayas *et al.*, 2004) remains controversial. For whilst stimulation
79 of the PVN modifies BP and HR (Kannan *et al.*, 1989; Martin *et al.*, 1991; Martin *et al.*, 1993; Duan *et*
80 *al.*, 1997; Schlenker *et al.*, 2001); others maintain that the PVN is not involved with the
81 cardiovascular response to stress itself (Stotz-Potter *et al.*, 1996; Fontes *et al.*, 2001; DiMicco *et al.*,
82 2002). One possible explanation for this is that since "stress" is a term which describes a wide range
83 of physiological and psychological stimuli, certain forms of stress (such as subcutaneous formalin,
84 (Culman *et al.*, 2010)) may activate tachykinin-mediated PVN responses, whereas others do not. It is
85 also possible that tachykininergic spinally projecting neurones may mediate other facets of
86 cardiovascular control. For example, PVN "pre-sympathetic" neurones have been implicated
87 circadian control of BP (Cui *et al.*, 2001).

88 In this work we use a specific saporin lesion of NK1 expressing PVN rat neurones with substance P-
89 saporin (SSP-SAP) and measure resting heart rate and heart rate variability (HRV) parameters in
90 response to mild psychological stress. We detected no change in overall daytime heart rate, or in
91 heart rate response to stress, but we find changes in daily heart rate rhythm and HRV response to
92 psychological stress. The HRV parameter "low frequency to high frequency ratio (LF/HF)" is often

93 used as an indicator of sympathetic activity and significantly increased with psychological stress. We
94 find the stress induced increase in this parameter to be blunted in the SSP-SAP lesion rats. We
95 conclude that NK1 expressing PVN neurones are involved with both the coupling of the
96 cardiovascular system to daily variations in heart rate and the sympathetic response to psychological
97 stress.

98

Methods

Ethical approval

All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 under a Home Office Licence. All surgery was performed under general anaesthesia as described in detail below.

Animals

All procedures were performed on young adult male Wistar rats (200-400g; n=6). Rats were maintained in the animal facility of the University of Liverpool on a 12-12 hour light-dark cycle. All animals had unlimited access to water and standard chow diet.

Immunofluorescence

Rats were terminally anaesthetised by intraperitoneal injection of Pentobarbitone (Pentoject, Animalcare, York, UK; 60 mg kg^{-1}) and perfused transcardially with 4% paraformaldehyde in PBS. Tissues were then removed and dehydrated with 30% sucrose in PBS overnight at $4^{o}C$ and 14 µm coronal cryostat sections prepared (Leica, UK). Immunofluoresence was performed using the primary antibody anti-rabbit Neurokinin-1 receptor (1:500; Abcam, UK) combined with the secondary antibody donkey anti-rabbit Dylight 594 (1:2000; Abcam, UK), and finally DAPI nuclei staining (0.1 µg/ml; Invitrogen). Cell counts were performed and efficacy of lesion was confirmed to be 100%.

Paraventricular nucleus of the hypothalamus - targeted injections of SSP-SAP

Specific lesions of the entire PVN were performed by injection of the cytotoxic Substance P-saporin (SSP-SAP) (0.04 mg/ml; Advanced Targeting Systems, San Diego, USA); a conjugation of saporin and

SSP, the Sar^{9}, $Met(O_2)^{11}$ analog of Substance P, shown to be selective in many studies (Khasabov & Simone, 2013; Talman & Lin, 2013).

Prior to surgery adult male Wistar rats (n=6; 200-400g) were put under isoflurane gas anaesthesia (4% v/v induction; 2% v/v maintenance) surgery was performed under aseptic conditions. Pre-operative subcutaneous injections of the analgesic buprenorphine (Temgesic, 1.5 mg/kg; Reckitt Benckiser, Slough, UK), the antibiotic enrofloxacin (Baytril, 0.2 ml/kg; Bayer AG, Leverkusen, Germany) and the anti-inflammatory meloxicam (Metacam, 100 µg/kg; Boehringer Ingelheim, Germany) were given. 50 nl SSP-SAP (n=3) or 50 nl PBS (control; n=3) were injected unilaterally in the right hand side gradually over a few minutes via a 5µl Hamilton syringe at previously defined PVN coordinates (1.8mm caudal, 1.8mm lateral, 9.2mm vertical at an angle of 10^{o}). These injections sites were based on the rat atlas and adjusted according to the size of the rat, site specificity was confirmed using immunofluorescence and dye injections (Figure 1) (Paxinos & Watson, 1986). The Hamilton syringe was left in the injection site for 5-10 minutes to avoid residual solution moving up the track from the syringe as much as possible.

Telemetry surgery, recording and mild stress handling

During lesion surgery electrocardiogram transmitters (ETA-F20; Data Sciences International, St Paul, MN, USA) were also implanted subcutaneously into rats under isoflurane gas anaesthesia. The rats were monitored postoperatively, and were allowed at least 7 days of recovery before any further procedures began. This recovery period was found to be sufficient for the re-establishment of normal HR patterns (Thireau *et al.*, 2008) and for the lesion to take effect. Rats were housed individually over receiver pads (Data Sciences International) and ECG recorded continuously. The ECG signal was digitized to a PC with a CED Micro1401 using Spike2 at 5 kHz. Heart rate was annotated using a custom program. Mild stress was induced by handling of the rats (Balcombe *et al.*, 2004) a few days after recording began.

144 *HRV analysis*

145 Heart rate variability analysis was performed using the Kubios HRV program (Niskanen *et al.*, 2004).
146 For power spectrum analysis, HR was resampled at 20Hz, and 3 min sections of clean and stable HR
147 were analysed by fast Fourier transform using Welch's periodogram with 50% overlapping windows
148 of 32 s. Low-frequency (LF) and high-frequency (HF) bandings were 0.15–1.0 and 1.0–5.0Hz,
149 respectively (previously verified by (Nunn *et al.*, 2013))

150 *Statistics*

151 Data was analysed by one-way ANOVA unless otherwise stated (Minitab). All data are presented as
152 means ± SEM. Power equations: we assumed a 6% SD of heart rate (Nunn *et al* 2013) and effect size
153 20%. A statistical power of 80% (α= 0.05) required two groups of 3 animals.

154

Results

Efficacy of Lesion

To confirm the action of the SSP-SAP lesion and the coordinates we have derived based on the stereotaxic rat atlas (Paxinos & Watson, 1986) we used immunofluorescence of the NK1 receptor on the PVN. As the lesion was unilateral the side which remained intact was used as a positive control. Figure 1A shows the intact side of the PVN, red staining indicates NK1-receptor staining, DAPI nuclear staining is blue. Figure 1B clearly shows the SSP-SAP lesioned side of the PVN; the lesion resulting in an absence of red NK1 receptor staining.

Effects of PVN NK1 lesion on 24hr heart rate

ECG was obtained in freely moving conscious rats using subcutaneous implantation of telemetric transmitters, and heart rate data was derived using a custom program. Daily variation in heart rate was plotted as average per 4 hours. Both control and lesioned animals showed increased heart rate at night compared to during the day (Figure 2); from 387 ± 6 to 423 ± 5 beats min^{-1} in control ($p<0.001$ by one way ANOVA; n=3 per group) and 399 ± 6 to 436 ± 5 beats min^{-1} in lesioned rats ($p<0.001$ by one way ANOVA; n=3 per group).

This data was fit with a standard sigmoidal waveform:

$$amp \times sin(2\pi ft + \varphi) + base$$

Where amp is the amplitude in bpm (i.e., the difference between maximum night time and minimum day time heart rate), f is the frequency in hr^{-1} (defined as 1/24), φ is the phase in radians and base is the baseline heart rate. There was a significant shift in the heart rate phase from 3.28 ± 0.16 to 4.49 ± 0.20 radians ($p<0.05$ Student's paired *t*-test Figure 2B); equivalent to a 3 hr shift in the cycle.

176 *Effect of lesion on cardiovascular response to psychological stress*

177 To determine the effect of mild psychological stress on cardiovascular parameters of NK1 receptor
178 PVN lesioned rats, the animals were subjected to mild handling stress. Activity as little as moving a
179 cage has been shown to increase heart rate and levels of the stress hormone corticosterone in the
180 plasma of rats (Seggie & Brown, 1975). Upon handling stress heart rate was seen to significantly
181 increase in a similar fashion in both control and lesioned rats (Figure 3A, 3B. 3C and 3D); 345 ± 2
182 beats min^{-1} to 414 ± 5 beats min^{-1} in control ($p<0.001$ by one way ANOVA; n=3 per group) and 354 ±
183 3 beats min^{-1} to 396 ± 11 beats min^{-1} in lesioned rats ($p<0.05$ by one way ANOVA; n=3 per group).
184 No significant difference in heart rate response to stress between the two groups was observed
185 (Figure 3D).

186 HRV analysis was performed on ECG recordings, as HRV is an indication of autonomic balance. The LF
187 to HF ratio (LF/HF) in particular, is a useful indicator of sympathetic *versus* parasympathetic balance.
188 Using power spectra analysis LF/HF was determined using previously validated frequency banding
189 (Nunn *et al.*, 2013) (Figure 4A and 4B). LF/HF was significantly increased in control rats from 0.84 ±
190 0.14 to 2.02 ± 0.15 (Figure 4C and 4D; $p<0.05$ by one way ANOVA; n=3 per group); indicating an
191 increase in sympathetic activity. This response was ablated in the SSP-SAP lesioned rats (Figure 4C
192 and 4D; $p>0.05$ by one way ANOVA; n=3 per group), suggesting a reduction in sympathetic drive due
193 to a loss of the NK1 expressing neurones.

194

Discussion

In this work, we show for the first time that PVN NK1 expressing neurones are involved with the daily variation of heart rate and also the sympathetic component of the response to mild psychological stress. Interestingly, the changes observed occurred after only unilateral lesion of the NK1 receptor-expressing neurones of the PVN. One may have expected compensation from the intact side to have nullified the effects of unilateral lesion. Two clear possibilities are (i) That the lesioning agent spread to the other side, however, this does not seem to be the case. In addition to sham controls, the unilateral lesion protocol allows the intact side to act as a control for the treated side, in terms of NK1 neurone ablation. We found that NK1 neurones were still present in the untreated side. An alternative hypothesis (ii) is that the effect would indeed have been much greater if both sides had been treated. For the present experiments, we treated one side only, partly so the intact side could act as an immunoflurescent control for the treated side (above) and partly because we were unsure as to what effect this treatment would have on the animals. Bilateral lesion may be a useful protocol to explore in future investigations of the role of NK1 receptors in the PVN.

A number of studies show conclusively that the PVN is important to cardiovascular control (Badoer *et al.*, 2002; Coote, 2005; Ramchandra *et al.*, 2013) and although others show the PVN to be central to the HPA component of the stress response (Herman & Cullinan, 1997; Herman *et al.*, 2002; Tavares *et al.*, 2009), the evidence that the PVN is directly involved in the sympathetic and cardiovascular stress response is less strong. Our own previous work shows that the spinally projecting "pre-autonomic" sympathetic PVN neurones express SP receptors and that these modulate the cardiovascular system (Womack *et al.*, 2007). Their mechanism of action is quite complex. SP interacts with the resting (tonic) inhibition of spinally projecting neurones by GABA (Womack *et al.*, 2007). This scheme involves change of the kinetic properties of spinally projecting

neurone GABAA receptors and is thus, presumably allosteric. Furthermore this cross-talk is PKC dependent (Yamada & Akasu, 1996).

One of the first studies to investigate the role of SP in cardiovascular response to stress used a combination of global NK1 knock-out and a selective, but blood brain barrier crossing, antagonist (intravascular) in mice. Whilst there was a clear reduction in heart rate increase to a noxious stimulus, it was not possible to determine where the active NK1 receptors were. Elevated plus maze experiments also showed a marked decrease in the behavioural attributes of stress when rats were given a specific NK1 receptor antagonist via ICV injection (Ebner *et al.*, 2008). Whilst this does not identify the location of the relevant NK1 receptors, this stressor also resulted in reduced c-Fos expression within the PVN of those rats treated with the NK1 receptor antagonist, implicating PVN NK1 receptors. Recent work by (Culman *et al.*, 2010) has also shown that ICV injection of specific tachykinin antagonists reduces the cardiovascular (and hormonal) response to stress, again these receptors could be anywhere accessible to the ICV injection. However, to investigate this further (Culman *et al.*, 2010) analysed the c-Fos response of PVN neurones in response to stress with and without tachykinin antagonist. They found the c-Fos response of corticotropin-releasing factor expressing PVN neurones was blunted by the tachykinin antagonists. This combination of studies therefore shows that NK1 receptors are involved with the cardiovascular and behavioural responses to severe (noxious) and psychological stress, and that NK1 receptors mediate at least a component of the response of PVN neurones by stress. However, we have now added one of the final pieces of data to this story by showing that reduction of NK1 expressing PVN neurones (by SSP-SAP unilateral lesion) mediates two specific facets of the LF/HF response to mild psychological stress. This type of heart rate variability analysis is often used as a method for quantifying the autonomic influence on the cardiovascular system based on HR variation over time. These natural rhythms occur at different frequencies associated with the sympathetic and parasympathetic nervous system influences. HRV is therefore widely used as an accurate indicator of autonomic balance (Malpas, 2002; Baudrie *et al.*,

2007; Thireau *et al.*, 2008) and autonomic response to stress (Farah *et al.*, 2006). Although there is no direct HRV indicator of sympathetic activity, a number of studies, including our own (Nunn *et al.*, 2013), have shown the LF/HR ratio is a valid measure of autonomic balance and therefore it is possible to infer changes in sympathetic activity using this parameter (Katoh *et al.*, 2002; Nunn *et al.*, 2013). In our previous study we methodically verified bandings for LF/HF boundaries and showed that atropine reduced the HF spectrum power and reserpine reduced the LF/HF ratio (Nunn *et al.*, 2013). Furthermore, in a previous study we directly showed that sympathetic activity of anaesthetised rats was stimulated by substance p (Womack *et al.*, 2007) in anaesthetised rats. We are therefore confident that our observed reduction of LF/HF power in freely moving rats does indeed indicate a genuine reduction of sympathetic activity.

We also found that PVN NK1 neurones are also involved with setting the daily variation of the rats' heart rate. Since the rats were kept under a 12hr light/12 hour dark cycle regimen, this could involve a changed behavioural reponse to conditions or it could suggest the involvement of these neurones in setting circadian cycles. Further experiments under fixed light conditions would be necessary to confirm the inherent hypothalamic rhythmicity has been affected rather than response to light itself. However, spinally projecting neurones of the PVN are involved with circadian rhythm. This was first suggested by (Cui *et al.*, 2001) who showed that spinally projecting neurones received input from the suprachiasmatic nucleus; a key centre of the hypothalamus involved with circadian rhythm (Reppert & Weaver, 2002). Neurones in this area have cyclically changing membrane potentials which allow general changes in activity on a 24 hour rhythm (Belle *et al.*, 2009). Studies show that this is paralleled by changes in rodent heart rate (Nunn *et al.*, 2013) and we find this involves PVN NK1 neurones, since their lesion significantly alters the rhythm, shifting it by approximately 3hrs. This is potentially of huge medical relevance, since in humans, hypertension is strongly linked to sympathetic activity (Mancia & Grassi, 2014) and circadian variation in cardiovascular control is

strongly linked to a spate of heart attacks that occurs in the morning (Muller *et al.*, 1989; Spielberg *et al.*, 1996; Lefer, 2010).

Our current data therefore provides urgently required data to show as directly as possible that the stress induced in sympathetic activity does involve PVN NK1 receptors and raises the possibility that potentially, selective inhibition of spinally projecting neurones could be therapeutically useful for modulation of stress related heart disease.

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Competing interests

The authors confirm there are no conflicts of interest.

Author contributions

Both authors have made substantial intellectual contributions to the conception and design of the study, data acquisition, analysis and interpretation. RBJ conceived the study and designed the experiments. CF designed and performed the experiments. Both contributed to data interpretation and manuscript preparation and approved the final version submitted.

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Figure Legends

Figure 1: Selective lesion of NK1 expressing neurones in rat PVN. (A) Unilateral injection with pontamine blue (1%). F=fornix. The dotted line indicates the approximate position of the PVN. Note that no dye crosses to the contralateral side. (B) Low magnification image of coronal section of PVN showing orientation using the 3rd ventricle. Intact side (left of the 3rd ventricle) and lesioned side (right of the 3rd ventricle), showing clear red staining for the NK1 receptor using the primary antibody anti-rabbit Neurokinin-1 receptor (1:500; Abcam, UK) combined with the secondary antibody donkey anti-rabbit Dylight 594 (1:2000; Abcam, UK) and blue DAPI nuclei staining (white arrows indicate staining). Scale bar is 100µm (C) Intact side of the PVN used as a positive control. Scale bar is 50µm (B) Lesioned side of the PVN from the same Wistar rat shows an absence of red NK1 receptor staining; blue DAPI nuclei staining remains. Scale bar is 50µm.

Figure 2: Daily variation in heart rate in SAP-SSP lesioned rats. (A) Circadian variation in heart rate was plotted as average heart rate per 4 hours in both control and SSP-SAP lesioned rats. This data was fit with a standard sigmoidal waveform and a significant shift in the circadian phase from 3.28 ± 0.16 to 4.49 ± 0.20 radians was observed ($p<0.05$ Student's paired *t*-test). **(B)** Control and lesioned rats both show increased heart rate at night compared to during the day; from 387 ± 6 to 423 ± 5 beats min^{-1} in control (n=3; $p<0.001$ by one way ANOVA) and 399 ± 6 to 436 ± 5 beats min^{-1} in lesioned rats (n=3; $p<0.001$ by one way ANOVA). No differences between the two groups were observed.

Figure 3: Heart rate response to stress in SAP-SSP lesioned rats. (A) Raw basal heart rate traces of control rats **(i)** before and **(ii)** after mild handling stress. **(B)** Raw basal heart rate traces of SSP-SAP rats **(i)** before and **(ii)** after mild handling stress. **(C)** Average heart rate per 5 minutes in both groups of rats. Arrow indicates time of mild handling stress. **(D)** Heart rate significantly increases both in control rats from 345 ± 2 to 414 ± 5 beats min^{-1} (n=3 per group; $p<0.001$ by one way ANOVA) and in

lesioned rats from 354 ± 3 to 396 ± 11 beats min^{-1} ($p<0.05$ by one way ANOVA; n=3 per group). No difference in heart rate response to stress between the two groups was observed.

Figure 4: LF/HF response to stress in SAP-SSP lesioned rats. (A) Representative fast Fourier transform for control rats **(i)** before and **(ii)** after mild handling stress. **(B)** Representative fast Fourier transform for SSP-SAP rats **(i)** before and **(ii)** after mild handling stress. In control animals an increase in LF and decrease of HF power is seen as a result of stress. Both LF and HF are reduced in lesioned rats after stress. **(C)** Average LF/HF ratio per 5 minutes in both groups of rats. Arrow indicates time of mild handling stress. **(D)** LF/HF ratio significantly increases in control animals subjected to stress from 0.84 ± 0.14 to 2.02 ± 0.15 (n=3 per group; $p<0.05$ by one way ANOVA). This response was abolished in SSP-SAP lesioned rats (n=3 per group; $p>0.05$ by one way ANOVA).